

Bacterial Infection of Wounds: Fibronectin-Mediated Adherence of Group A and C Streptococci to Fibrin Thrombi In Vitro

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Adherence of group A, B, and C streptococci to fibrin thrombi was studied by using a novel fluorochrome microassay carried out in microdilution plates in which fibrin thrombi had been prepared by clotting citrated human, cattle, or horse plasma. Substantial adherence was observed with various strains of group A and C streptococci, whereas little was observed with group B streptococci. Adherence of all group A and C streptococcal strains decreased by up to 40% when fibronectin was depleted from the plasmas used for preparing fibrin thrombi, and fibronectin repletion increased adherence of streptococci in a dose-dependent manner. Addition of the 210-kilodalton C-terminal fragment of fibronectin to fibronectin-depleted plasma restored the adherence of group C but not group A streptococci, whereas addition of the 29-kilodalton N-terminal fragment was without any effect for all tested streptococcal strains. Prior incubation of group A and C streptococcal strains with fibronectin markedly increased their adherence, but treatment with proteases abolished their ability to adhere to fibrin thrombi. Adherence of group B streptococci was not affected by either fibronectin depletion or proteolytic digestion. These results indicate that both fibronectin incorporated into the fibrin matrix of thrombi and soluble fibronectin can mediate adherence of group A and C streptococci to fibrin thrombi and that binding sites for fibronectin located on the bacterial surface mediate this adherence.

The first step in bacterial invasion of eucaryotic tissue is the attachment of bacteria to host cell surface. This attachment involves specific interactions between components of the bacterial cell wall and complementary structures on the host cell surface (1). Fibronectin, an adhesive plasma protein which is present on many mammalian cell types (24), has been reported to be one of the components mediating adherence of staphylococci and streptococci to various cell types of the host organism (27, 29, 31, 40). Although the adherence of streptococci to these cells has been well studied, the precise mechanism of attachment to skin wounds which leads to colonization of the wound is still unknown. Fibronectin is localized in the fibrin matrix over which the epidermis migrates during wound healing (10). The adhesive properties of fibronectin are of importance in the process of wound healing since fibronectin mediates the attachment of fibroblasts, epithelial cells, and monocytes to an injured site (2, 13, 16). In healing wounds and the area of inflammation, plasma fibronectin is deposited as part of a blood clot (14, 15) and accumulates during increased vascular permeability (9). Fibronectin is also produced by cells of blood vessels in response to injury (11, 25). Skin wounds are important entry sites for bacteria to start an infection (19). Bacterial adhesion is the most important factor in the initial phase of development of skin wound colonization (22). Interaction of molecules on the bacterium and host tissues can lead to colonization of traumatized tissues and blood clots (41). Since many species of streptococci bind fibronectin (5, 26), it was of interest to investigate the role of both incorporated and soluble fibronectin in adherence of streptococci to fibrin thrombi. For such studies, it was

helpful to have an in vitro method for precise quantification of streptococcal adherence to fibrin thrombi. The bioassays (12, 22) involve use of experimental animals and lack the possibility of altering various parameters required for determination of the mechanism of bacterial attachment. In the present study, we describe a novel fluorochrome microassay for determination of adherence of streptococci to fibrin thrombi and a possible mechanism involved in this adherence. Using this method, we studied the adherence of human pathogenic *Streptococcus pyogenes*, cattle pathogenic *S. dysgalactiae*, and horse pathogenic *S. equi* to fibrin thrombi prepared from plasmas of the respective host species.

MATERIALS AND METHODS

Streptococci. A total of 25 streptococcal strains were used. Of these, 10 belonged to *S. pyogenes* (serological group A) and 5 each belonged to *S. dysgalactiae* and *S. equi* (both of serological group C). Group A strains included DSM 2071 (ATCC 21059), DSM 2072 (ATCC 21060), DSM 20565 (ATCC 12344, NCTC 8198), DSM 2073, DSM 2074, and five clinical isolates. *S. dysgalactiae* strains included DSM 20662 (ATCC 43078), NCTC 4335, NCTC 4669, and two clinical isolates from mastitis of cattle. *S. equi* strains included DSM 20561 (ATCC 33398, NCTC 9682), DSM 20727 (ATCC 43097, NCTC 7023), and three clinical isolates. Five cultures of *S. agalactiae* (serological group B), including strains DSM 2134 (ATCC 13813, NCTC 8181) and NCTC 11360, served as controls since they did not interact with fibronectin, as determined in preliminary experiments (6). Five independent isolates of *Staphylococcus aureus* from human infections were also included in adherence assays. The bacteria were cultivated in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, England) and harvested by centrifugation (20 min, 15,000 × g). After a wash with 0.1 M sodium bicarbonate-carbonate, pH 9.2, the bacteria were suspended in the same buffer and adjusted to 10⁹ bacteria per ml by optical density at 600 nm.

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The bacteria were then labeled with fluorescein isothiocyanate (Sigma, Deisenhofen, Federal Republic of Germany [FRG]) as described previously (36–38) and suspended in 0.15 M phosphate buffer, pH 7.5, containing 150 mM NaCl (phosphate-buffered saline).

Fibrin thrombi. Fibrin thrombi were prepared by clotting citrated plasma from humans, cattle, or horses. For this, 50 μ l of plasma was mixed with 25 μ l of the clotting reagent in each well of a 96-well black microdilution plate (Flow Laboratories, Meckenheim, FRG). The clotting reagent consisted of 0.25 U of thrombin (Sigma) in 0.125 M CaCl_2 –0.15 M NaCl. Clotting of plasma was carried out for 5 min at room temperature followed by 30 min at 37°C. The thrombi were washed two times with 0.15 M NaCl before the adherence assay was performed.

Adherence assay. The adherence assay was performed directly in the microdilution plates in which fibrin thrombi had been prepared. Adherence was started by adding 100- μ l aliquots of streptococcal suspension containing 10^8 bacteria in each well. After 30 min at 37°C under 5% CO_2 , the fibrin thrombi were washed three times with 0.15 M NaCl to remove nonadherent bacteria. The fluorescein associated with adherent streptococci was then measured in a microdilution plate fluorescence reader (Fluoroskan II; Flow Laboratories). Results were expressed as the number of adherent streptococci, which was calculated from the absolute fluorescence value of the total number of streptococci in each assay. Any possible growth of bacteria during the 30-min assay was not taken into consideration in calculating the number of adherent streptococci. Each test was performed in triplicate. In control experiments streptococci were replaced by phosphate-buffered saline.

Fibronectin and fragments. Human, bovine, and equine fibronectins were purified by affinity chromatography on gelatin-agarose and heparin-agarose (5, 23). The purity of fibronectin preparations was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (21) and Western blotting (immunoblotting) (34). For binding studies, fibronectin preparations were labeled with ^{125}I , using the chloramin-T method (18). For preparation of fibronectin fragments, 50 mg of fibronectin was treated with 750 U of thrombin (Sigma, Munich, FRG) for 24 h at 37°C (5, 17). A 210-kilodalton (kDa) C-terminal fragment was purified from the digest by affinity chromatography on gelatin-agarose followed by gel filtration on a Superose 6 column using high-performance chromatography (FPLC; Pharmacia, Freiburg, Federal Republic of Germany). A 29-kDa N-terminal fragment was purified from thrombin digest by heparin-agarose chromatography followed by filtration on a Superose 12 column using high-performance chromatography (FPLC; Pharmacia). Both fragments were analyzed for their homogeneity and N-terminal amino acid residues as described previously (5).

Determination of role of fibronectin in adherence. Plasmas were depleted of fibronectin by repeated passage through a gelatin-agarose column. The effluent plasmas were free of fibronectin as determined by their negative reactions with antibodies against fibronectin in immunodiffusion and dot blot analysis (32). Adherence assays were then performed in parallel, using normal plasma, fibronectin-depleted plasma, and plasma to which fibronectin had been supplied at concentrations of 1.2 to 150 $\mu\text{g}/100 \mu\text{l}$ of plasma. Purified fibronectin preparations from the homologous host species were used for repletion. In some experiments, repletion was with fibronectin fragments instead of fibronectin. Incorporation of fibronectin or its fragments into clots was determined

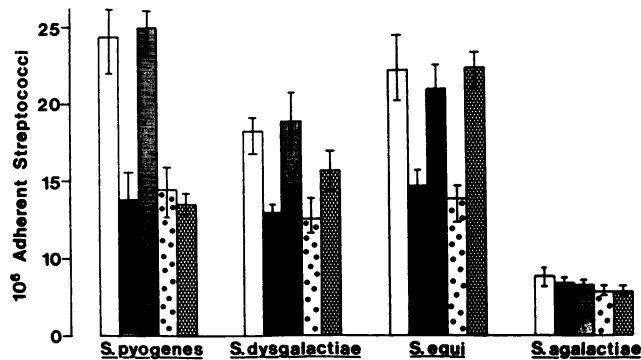


FIG. 1. Adherence of streptococci to fibrin thrombi prepared from normal plasma (open bars), fibronectin-depleted plasma (striped bars), fibronectin-free plasma supplemented with fibronectin (shaded bars), 29-kDa fragment of fibronectin (spotted bars), and 210-kDa fragment of fibronectin (cross-hatched bars). Plasma and fibronectin from humans, cattle, and horses were used for *S. pyogenes*, *S. dysgalactiae*, and *S. equi* strains, respectively. Adherence of *S. agalactiae* strains was determined with both human and bovine fibrin thrombi. The final concentration of fibronectin and the fragments in repleted plasma was 0.68 μM . Values are expressed as number of adherent streptococci per fibrin thrombus as mean and range of 10 *S. pyogenes* strains and five strains each of other streptococcal species.

by addition of 0.68 μM protein containing 100,000 cpm of ^{125}I -labeled respective protein. The clots were then washed thoroughly, and the incorporated radioactivity was determined in a gamma counter. In further experiments, the role of soluble fibronectin was analyzed by prior treatment of streptococci with increasing concentrations (0.4 to 50 $\mu\text{g}/10^8$ streptococci) of fibronectin.

Proteolytic treatment of streptococci. Aliquots of streptococcal suspensions were incubated with increasing (2 to 250 $\mu\text{g}/\text{ml}$) concentrations of pronase or trypsin (Merck, Darmstadt, FRG) as previously described (4, 7, 8). The bacteria were then washed five times with phosphate-buffered saline and used in the adherence assay.

RESULTS

Adherence of streptococci to fibrin thrombi. Determination of streptococcal adherence to fibrin thrombi in 96-well microdilution plates in which the fibrin thrombi had been prepared proved to be highly convenient, rapid, quantitative, and flexible with respect to changing plasma composition and allowed measurement of a large number of samples simultaneously. Various strains of *S. pyogenes*, *S. dysgalactiae*, and *S. equi* adhered to fibrin thrombi with mean values of 2.5×10^7 , 1.7×10^7 , and 2.2×10^7 adherent streptococci per thrombus, respectively. The presence of serum albumin (1 mg/ml) in the assay buffer had no influence on the adherence of streptococci. Viability of streptococci was not required for adherence to fibrin thrombi. Heat-killed streptococci (1 h at 80°C) adhered up to 70% as compared with normal streptococci. Group B streptococci, which did not bind fibronectin, showed a relatively weak (6×10^6 bacteria per thrombus) adherence to fibrin thrombi (Fig. 1).

Role of fibronectin in streptococcal adherence. Labeling of streptococci with fluorescein isothiocyanate did not alter their fibronectin binding as shown by binding studies with ^{125}I -labeled fibronectin, using fluorescein isothiocyanate-labeled and unlabeled streptococci in parallel. Adherence of streptococci to thrombi which had been prepared from

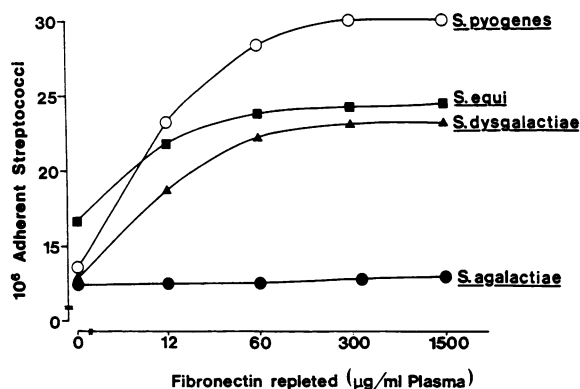


FIG. 2. Adherence of streptococci to fibrin thrombi prepared from fibronectin-free plasma supplemented with various concentrations of fibronectin. Plasma and fibronectin preparations of the respective host species were used. Each point is a mean value of 10 *S. pyogenes* strains and five strains each from other streptococcal species.

fibronectin-depleted plasma was up to 40% lower compared with that to normal fibrin thrombi. This decrease was most substantial with *S. pyogenes* strains (mean, 40%) but moderate with *S. dysgalactiae* and *S. equi* strains (mean, 24 and 32%, respectively). Adherence of group B streptococci, which served as controls, was not affected by depletion of fibronectin (Fig. 1). The adherence of *Staphylococcus aureus* strains to fibrin thrombi also was not affected by depletion of fibronectin. Fibrin thrombi prepared from plasma that had been depleted of fibronectin and then supplied with purified fibronectin bound streptococci as efficiently as those prepared from normal plasma. Adherence of group B streptococci as well as of *Staphylococcus aureus* strains was not affected by repletion of fibronectin (Fig. 1). Addition of fibronectin at concentrations of 0.012 to 1.5 mg/ml of plasma increased streptococcal adherence in a dose-dependent manner (Fig. 2).

Role of fibronectin fragments. To identify the domain of fibronectin involved in mediation of adherence of streptococci to fibrin thrombi, fibronectin-free plasma was supplied with equimolar concentrations of fibronectin and 29-kDa N-terminal and 210-kDa C-terminal fragments of fibronectin. An incorporation assay using radiolabeled proteins showed that between 92 and 95% of fibronectin and its fragments were incorporated into the fibrin clot. Repletion with the 29-kDa fragment failed to increase the adherence of any tested species of streptococci to fibrin thrombi. Repletion with the 210-kDa fragment increased the adherence of *S. dysgalactiae* and *S. equi* strains but did not affect the adherence of *S. pyogenes* (Fig. 1).

Role of soluble fibronectin in streptococcal adherence. Prior incubation of streptococci with increasing concentrations (0.4 to 50 $\mu\text{g}/10^8$ streptococci) of purified fibronectin preparations increased streptococcal adherence to fibrin thrombi in a dose-dependent manner. Adherence of group B streptococci was not affected even after preincubation with 50 μg of fibronectin per 10^8 streptococci (Fig. 3).

Effects of proteolysis of streptococci on adherence. Adherence of all *S. pyogenes* strains to fibrin thrombi was strongly inhibited when the bacteria were pretreated with pronase or trypsin, both of which are known to destroy the fibronectin-binding sites of *S. pyogenes* (5, 6). Similarly, the adherence of *S. dysgalactiae* and *S. equi* strains was inhibited by pronase treatment. The inhibition by trypsin, however, did

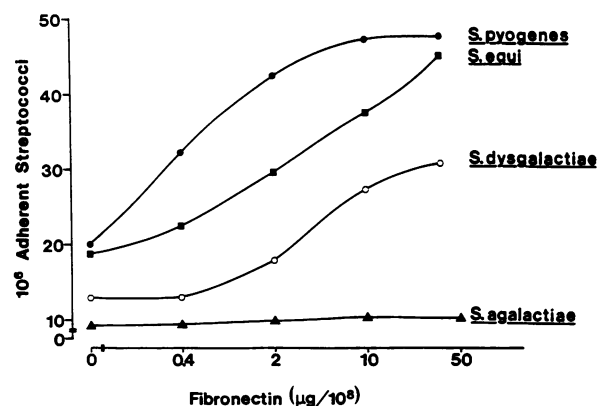


FIG. 3. Effect of soluble fibronectin on adherence of streptococci to fibrin thrombi. Ten strains of *S. pyogenes* and five each of *S. dysgalactiae*, *S. equi*, and *S. agalactiae* were preincubated with various concentrations of fibronectin and washed before determination of their adherence to fibrin thrombi. Each point is a mean from all tested strains of respective streptococcal species.

not exceed 50% even at concentrations of 250 $\mu\text{g}/10^8$ streptococci (Fig. 4).

DISCUSSION

Conflicting results have been reported on the involvement of fibronectin in the mediation of bacterial attachment to wound surfaces. The study by Mertz et al. (22) indicated that adherence of *Staphylococcus aureus* to skin wounds obtained from experimental animals was not mediated by fibronectin. On the other hand, Toy et al. (35) reported the involvement of fibronectin in attachment of *Staphylococcus aureus* to fibrin clots. These conflicting results could have been due to the use of different methods, neither of which gave precise quantification of adherent bacteria. The development of a quantitative method to determine the number of adherent bacteria is a prerequisite for elucidation of the role of fibronectin in adherence of bacteria to fibrin thrombi and subsequently in wound infection. The method described here is simple and rapid and allows quantification of adher-

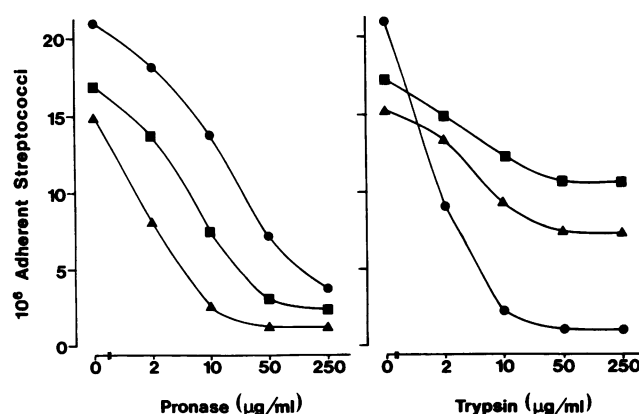


FIG. 4. Effect of proteolytic digestion of streptococci on their adherence to fibrin thrombi. Different strains of *S. pyogenes* (●), *S. dysgalactiae* (▲), and *S. equi* (■) were treated with different concentrations of pronase or trypsin before being used in adherence assays. Each point is the mean of five strains of each streptococcal species.

ent bacteria; moreover, it does not involve the use of experimental animals. By allowing parameters to be precisely altered at will, the method permits identification of components that play a role in adherence and thus elucidation of adherence mechanisms.

Using the method, we observed that group A and C streptococci, both known to bind fibronectin (5, 20, 33), adhered substantially more to normal fibrin thrombi than to those which had been prepared from fibronectin-depleted plasma. Repletion of such plasmas with fibronectin increased streptococcal adherence, confirming the involvement of fibronectin in the attachment process. Group B streptococci which do not interact with fibronectin adhered equally poorly to normal, fibronectin-depleted, or fibronectin-supplemented fibrin thrombi. Serum albumin, which has specific receptors on certain streptococci (42) and the ability to alter fibronectin interaction with certain bacteria (39), had no influence on the adherence of streptococci to fibrin thrombi. This is consistent with our previous findings (6) which showed no effect of albumin on the binding of fibronectin to streptococci. To determine the domain of fibronectin involved in adherence to fibrin clots, we made use of two fibronectin fragments, a 29-kDa N-terminal and a 210-kDa C-terminal fragment. These fragments were chosen because each possesses a single fibrin-binding domain (28). The 29-kDa fragment failed to mediate adherence of any tested streptococci, whereas the 210-kDa fragment could substitute for fibronectin in adherence of *S. dysgalactiae* and *S. equi* but not of *S. pyogenes*. This might be explained by the fact that *S. pyogenes* binds fibronectin at its N-terminal domain whereas *S. dysgalactiae* and *S. equi* bind through a domain in the C-terminal part of the molecule (5). The data presented here suggest that binding sites for *S. pyogenes* in the 29-kDa N-terminal fragment are not available once the fragment is incorporated in the fibrin clot. Thus, the domain of fibronectin required for mediation of adherence of human pathogenic *S. pyogenes* is different from the animal pathogenic *S. dysgalactiae* and *S. equi*. Although both *S. pyogenes* and *Staphylococcus aureus* interact with the 29-kDa N-terminal domain of fibronectin, the adherence of only *S. pyogenes* was mediated by fibronectin. This might suggest that the 29-kDa N-terminal fragment of fibronectin has different binding domains for *S. pyogenes* and *Staphylococcus aureus*. As reported by Mertz et al. (22), fibronectin was unable to mediate the adherence of *Staphylococcus aureus* to wounded tissue. Therefore, different mechanisms might exist for adherence of *Staphylococcus aureus* to fibrin clots on the one hand and to eucaryotic cells (40) and incorporated foreign bodies (39) on the other hand.

Fibronectin binding sites on streptococci were essential for fibronectin-mediated adherence of streptococci to fibrin thrombi. Proteolytic digestion of streptococcal binding sites for fibronectin with trypsin or pronase blocked streptococcal adherence to fibrin thrombi. The different efficacies of the two proteolytic enzymes in inhibiting adherence corresponded well with previously determined proteolytic sensitivities of fibronectin binding to group A and C streptococci (6). Moreover, heat killing of streptococci led to about a 30% decrease in adherence, which is consistent with the corresponding loss of fibronectin binding activity after heat treatment (6).

To determine the consequence of streptococcus-fibronectin-fibrin interaction in wound infection, it is important to consider the role of soluble fibronectin. Inflammatory fluid present in wounds also contains soluble fibronectin which might inhibit the binding of streptococci to fibronectin incor-

porated in the thrombi. In this case, the bacteria might be confined to the external surface of the wound and their further penetration of the wounded tissues might be diminished (3). Our results indicate, however, that soluble fibronectin not only did not inhibit streptococcal adherence to fibrin thrombi but increased it more than twofold. This may be explained by the fact that, unlike the situation with fibronectin receptors on epithelial or endothelial cells, the fibrin clot might possess a large proportion of unoccupied fibronectin binding sites that provide adherence opportunities for bacteria that had already soluble fibronectin. There might be some difference in streptococcal affinity for soluble and immobilized fibronectin which would explain the higher adherence observed with soluble fibronectin.

The results of our study show that both soluble and immobilized fibronectin can mediate streptococcal adherence to fibrin thrombi and suggest that the streptococcal binding sites for fibronectin may represent an important bacterial component of the mechanism of initiation of wound infection.

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